Short Communication

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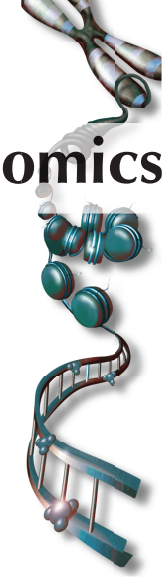
Epigenomics



Methylome of skeletal muscle tissue in patients with hypertension and diabetes undergoing cardiopulmonary bypass

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Background: Epigenomic changes occurring during surgery have been neglected in research; diabetes and hypertension can affect the epigenome but little is known about the epigenetics of skeletal muscle (SKM). **Methods:** DNA methylation was profiled via Illumina MethylationEPIC arrays in SKM samples obtained at the beginning and end of heart surgery with cardiopulmonary bypass. **Results:** Methylation in patients with hypertension and diabetes was significantly different, more so for uncontrolled diabetes; hypertension alone produced minimal effect. The affected pathways involved IL-1, IL-12, IL-18, TNF-α, IFN-γ, VEGF, NF-κB and Wnt signaling, apoptosis and DNA damage response. Significant changes occurred during surgery and included loci in the Hippo–YAP/TAZ pathway. **Conclusion:** Cardiopulmonary bypass surgery affects the SKM methylome, and the combination of hypertension and diabetes induces changes in the SKM epigenome in contrast to hypertension alone.

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Keywords: cardiac surgery • cardiopulmonary bypass (CPB) • diabetes • DNA methylation • epigenomics • hypertension • skeletal muscle

Epigenetics is a powerful regulatory mechanism that plays a number of roles in health and disease [1]. Surgery has been linked to epigenetic alterations in tissues and cells that may mediate physiological and pathological gene expression changes which may affect postoperative recovery across a wide array of processes, ranging from wound healing to pain perception [2–7]. Nevertheless, the paucity of reports on the subject contributes to a lack of detail in our knowledge of surgery-induced epigenetic effects. Here we present a 'pilot' report on the epigenome-wide DNA methylation profiles of skeletal muscle (SKM) tissue specimens obtained at the beginning and end of cardiopulmonary bypass (CPB) procedures.

Diabetes mellitus (DM) and hypertension (HTN) are major risk factors for cardiovascular diseases and major causes of morbidity/mortality in the USA [8]. Given the association of DM and HTN with cardiovascular disease and worse cardiac surgery outcomes [9,10], the patients included were those with HTN, either alone or in combination with DM, and controls who did not have either disease. While other components of the 'metabolic syndrome' have been linked to epigenetic changes in SKM, albeit without providing a full view of the epigenomic landscape [11,12], we found no reports on the effects of HTN on the muscle epigenome. We hypothesized that the regulatory changes in SKM that develop in patients with HTN and DM include epigenetic regulation, and aimed to characterize the epigenome-wide signature of these changes, both at baseline and after cardiac surgery.

Patients & methods

Patients

Patients underwent coronary artery bypass grafting and/or valve replacement at Rhode Island Hospital. Informed consent was obtained in accordance with protocols approved by the Rhode Island Hospital IRB (no. 225612–53, 18 May 2021). The patients comprised of four study groups with four patients per group: 'control' (neither HTN nor

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DM), 'HTN' alone, HTN plus controlled DM ('HTN+cDM') or HTN plus poorly controlled or uncontrolled DM ('HTN+uncDM'); the total number of samples (one before and one after surgery for each patient) was therefore 32. Patient characteristics are listed in Supplementary Tables 1 & 2.

The patients underwent an open chest cardiac surgery with CPB involving general anesthesia, systemic heparinization (activated clotting time >400 s), aortic cross clamp and use of a blood cardioplegia perfusion system. Two specimens 5 cm apart in the region of the left internal mammary artery bed (innermost intercostal muscle) were obtained firstly after sternotomy and prior to cannulation (immediately before CPB onset) in the region of the internal mammary artery bed, and secondly after separation from CPB and decannulation. Specimens were immediately frozen in liquid nitrogen.

Diagnosis criteria

Patients with HTN and DM were identified based on clinical diagnosis in their electronic medical record. Clinical diagnosis of HTN was achieved using the most recent American College of Cardiology and American Heart Association guidelines: two separate recordings of systolic blood pressure ≥130 mmHg [13]. DM was diagnosed with a clinical diagnosis and a fasting serum glucose >126 mg/dl and HbA1c >6.5%. The most recent HbA1c within the past 3 months was used to determine whether the patient's DM was controlled or uncontrolled. Patients with HbA1c ≤ % had controlled DM and those with HbA1c >7% had poorly controlled or uncontrolled DM.

DNA samples

A 30-mg SKM sample was excised by scissors and ground using a probe homogenizer in Qiagen (Hilden, Germany) lysis buffer then lysed overnight at 56°C. The lysates were subjected to Qiagen DNeasy® procedure (Qiagen) with RNAse treatment. Quality and quantity of the DNA were determined spectrophotometrically (Implen, CA, USA). The samples were submitted for hybridization at Mass General Brigham Biobank Genomics Core using Illumina (CA, USA) Infinium® MethylationEPIC BeadChip Arrays.

Data analysis

Raw data (.idat) files were imported via Illumina Genome Studio (v. 2011.1 with methylation module 1.9; Illumina) for quality control, and it was determined that the performance of samples and chips, including hybridization and bisulfite conversion, were satisfactory. The data files were then imported into Partek Genomics Suite (v. 7.19; MO, USA) for analysis. X and Y chromosome loci were excluded; hence the analysis only includes autosomal loci. We used Infinium MethylationEPIC v. 1.0 B5 manifest and MethylationEPIC v. 1.0 B4 annotation files. We used 'functional normalization', which is a Bioconductor minfi package normalization, with NOOB background correction (out-of-band normalization) and dye correction [14,15]. The resultant β-values for 846,232 loci were converted into M-values [16]. The β-value reflects percentage methylation, and ranges from 0–1; the M-value is a derivative of the β-value that is more suitable for biostatistical transformations.

We performed data quality control by analyzing sample box plots and methylation β-value and M-value distribution histograms. Group analysis was performed via principal component analysis (PCA). Comparisons between groups to identify differentially methylated loci (DML) were performed using analysis of variance (ANOVA) over M-values using a false discovery rate (FDR)-adjusted $p < 0.05$ threshold [17,18]. The Partek Suite incarnation of ANOVA performs group-by-group comparisons using Fisher's least significant differences post-test; we used either a stringent approach – FDR-adjusted $p < 0.05$ threshold plus a difference value of 2(-2) (i.e., greater or smaller by a value of 2) – or a relaxed approach with unadjusted $p < 0.05$ or $p < 0.01$, as detailed in the Results section. In some cases (as detailed in Results), we performed pairwise comparisons using a paired t-test. The comparisons are illustrated by hierarchical clustering (HCL) analysis. Additionally, in some cases (as detailed below) we performed linear models for microarray data (LIMMA) analysis via TIGR MeV (mev.tm4.org) 4.5.1 or 4.8.

Network analysis and pathway enrichment were performed via GeneGo Metacore (Clarivate, PA, USA). The CpGs in a list of DMLs were annotated by the nearest gene using Illumina annotation manifest, then an outcome list containing gene names was used for network and pathway analyses, which were performed on the basis of gene names. Metacore contains a large manually curated database of known factor-to-factor interactions. Network design used a 'direct interactions' algorithm which builds a graphical network of factors from a list connected by interactive lines between them, each depicting a published interaction between the two factors.

Pathway mapping in Metacore relies on pre-designed graphical maps of specific pathways which form the pathway database. The algorithm determines what genes (factors) in the source list also occur in these pathways and selects the top pathways enriched.

Raw data are available as NCBI GEO GSE173613.

Results

Preoperative samples

We first analyzed preoperative samples separately to determine the effects of HTN and DM on DNA methylation in SKM.

The sample box and whiskers chart indicates that, overall, methylation across samples was reasonably distributed (Supplementary Figure 1). The histograms of β-values and M-values show a typical distribution (Supplementary Figure 2).

Principal components analysis

PCA (Figure 1) indicated that the samples in the HTN+cDM and HTN+uncDM groups had lower variability, while samples in the control and HTN only groups showed greater variability within their respective groups. The findings from the PCA suggest that the effects of DM are more pronounced than the effects of HTN alone on DNA methylation.

Analysis of variance

We performed ANOVA over M-values using a $p < 0.05$ FDR threshold with Fisher's least significant differences post-test. The comparison of control versus HTN only returned only three loci with a significant difference in DNA methylation. In contrast, at the same stringency the numbers of loci with a significant difference in DNA methylation were: 5823 in control versus HTN+cDM, 7177 in control versus HTN+uncDM, 2594 in HTN versus HTN+cDM, 3006 in HTN versus HTN+uncDM and 0 in HTN+cDM versus HTN+uncDM. The lists 'control versus HTN+cDM' and 'control versus HTN+uncDM' were 77% identical, while the lists for HTN versus HTN+cDM and HTN versus HTN+uncDM were 68% identical. Notably, 80% of the DMLs on the HTN versus HTN+uncDM list were present in the 'control versus HTN+uncDM' list (Supplementary Figure 3). All four major comparisons in Supplementary Figure 3 had 1535 overlapping loci. Of these loci, 191 were mapped to 5′ UTR or first exon, 23 to 3′ UTR, 159 to first exon, 292 to gene body, 252 to <1500 bp from transcription start site, and the others to nearby intergenic locations.

These results suggest that HTN alone had a minimal effect on DNA methylation, whereas addition of DM (whether controlled or uncontrolled) led to broad effects. There were no significant loci between HTN patients with controlled versus uncontrolled DM. Nevertheless, the combination of HTN and DM induced more methylation changes than HTN alone, suggesting that HTN potentiates epigenomic effects of DM but has minimal effects on its own.

We performed HCL analysis for each list of significant loci (Figure 2). Each of these HCL heat maps is derived from a list comparing two groups in the post-ANOVA test as indicated in the title; sample values from the other two groups 'tag along' for illustrative purposes. These profiles demonstrate the dramatic effects of DM (whether controlled or uncontrolled) as compared with the effects of HTN alone.

LIMMA

In order to narrow down the significant loci, we filtered the data by overall ANOVA p-value (0.05) and obtained a matrix of 72,318 loci. We then performed a LIMMA analysis across the four groups with $p < 0.05$, $p < 0.01$ and $p < 0.001$ thresholds in TIGR MeV 4.5.1.

This approach also demonstrated that there were minimal changes in control versus HTN only as well as HTN+cDM versus HTN+uncDM groups. The findings also suggest that the effects of DM on DNA methylation are major (Figure 2F).

Pathway analysis

We used LIMMA outputs to perform pathway enrichment in GeneGo Metacore. We used three of the gene lists, with results as follows: control versus HTN+cDM yielded 2724 loci resulting in 2067 annotated objects; control versus HTN+uncDM yielded 2845 loci resulting in 2197 annotated objects (these two lists were 77%

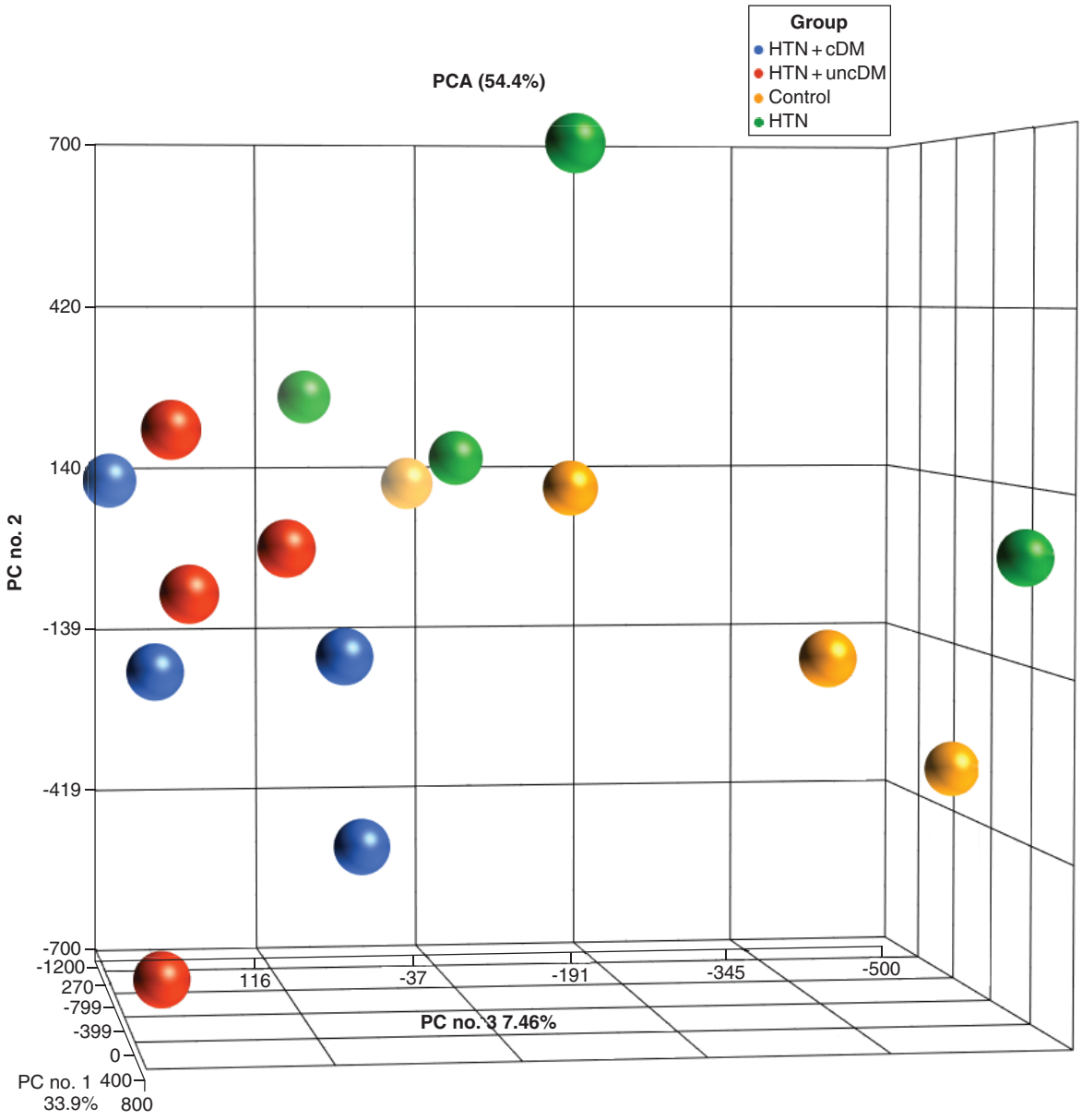

Figure 1. Principal components analysis of preoperative samples. The figure illustrates similarity or dissimilarity of DNA methylation between samples. Principal components analysis shows the methylation profiles of the study samples. Each sample is represented by a dot, the axes are first three principal components and the number in parentheses indicates the fraction of variance explained by each component. The samples are colored by study group. cDM: Controlled diabetes mellitus; HTN: Hypertension; PC: Principal component; PCA: Principal component analysis; uncDM: Uncontrolled diabetes mellitus.

identical); and HTN versus HTN+uncDM yielded 879 loci resulting in 748 annotated objects (this list was 73% identical with HTN versus HTN+cDM [987 loci]). Notably, 96% of the significant loci in the HTN versus HTN+uncDM list were also present in the control versus HTN+uncDM list. In other words, the list 'control versus HTN+uncDM' was the largest of all comparisons, and it best represents the loci affected by HTN and DM.

Network analysis of the 'control versus HTN+uncDM' list of loci showed that the genes associated with the altered loci form a tightly interacting network with 560 hubs and 7055 edges (Supplementary Figure 4). The other group comparisons are not shown due to space constraints as well as a high extent of similarity between the lists of loci. The network analysis indicates that the affected genes interact directly with each other to a great extent in biological processes.

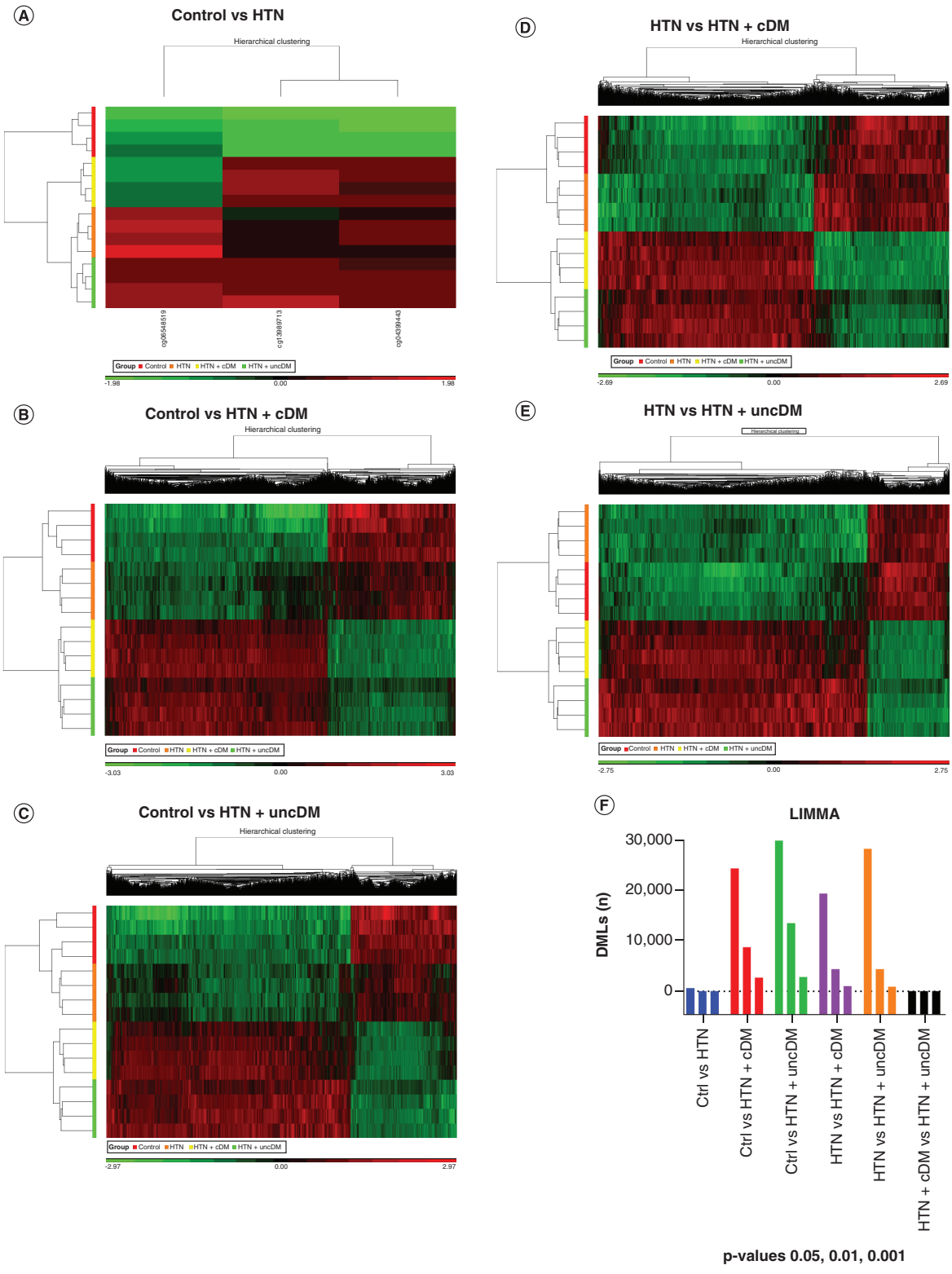

Figure 2. Analysis by hierarchical clustering and linear models for microarray data. (A–E) Hierarchical clustering of analysis of variance post-tests comparing two groups, as indicated. **(A)** Control versus HTN. **(B)** Control versus HTN + cDM. **(C)** Control versus HTN + uncDM. **(D)** Hypertension versus HTN + cDM. **(E)** HTN versus HTN + uncDM. In each clustering dendrogram, sample values from the other two groups 'tag along' for illustrative purposes. Values were row-normalized. Green: low methylation; red: high methylation. **(F)** Linear models for microarray (LIMMA) results. The significantly different loci were filtered by overall analysis of variance p-value (0.05) and the matrix of 72,318 loci was subjected to LIMMA analysis over four groups with $p < 0.05$, $p < 0.01$ and $p < 0.001$ thresholds. The chart represents the number of differentially methylated loci in these comparisons.
cDM: Controlled diabetes mellitus; Ctrl: Control; HTN: Hypertension; uncDM: Uncontrolled diabetes mellitus.

Pathway analysis identified several key processes, including protein folding and maturation, immune and inflammatory response (IL-1, IL-12, IL-18, TNF-α, IFN-γ, VEGF and NF-κB signaling), growth (Wnt signaling), apoptosis and cellular response to DNA damage. GoProcess annotation revealed that the major effects were focused on metabolic processes: protein synthesis and regulation of cell cycle. Names and p-values for the top 50 pathways and processes involved are shown in Supplementary Figure 5.

Overall, our findings suggest that HTN alone did not induce major changes in DNA methylation in SKM. However, HTN combined with DM, whether controlled or uncontrolled, induces substantial changes that involve several important inflammatory, immune and metabolic pathways.

Relaxed pairwise comparisons

Because multiple group comparisons were used with stringent criteria, it is possible that the results omit some of the effects that could be identified in more relaxed pairwise comparisons. Hence only 'control versus HTN' sample data were imported, and a t-test over normalized M-values was performed to obtain unadjusted p-values. At $p < 0.01$ unadjusted, 3451 DMLs were detected. Similarly, HTN+cDM and HTN+uncDM samples were separately imported and 8360 DMLs were detected. However, comparison of control and HTN+uncDM samples led to the detection of 17,124 DMLs, and comparison of HTN versus HTN+uncDM produced 10,477 significant DMLs. These findings provide further support that DM had more pronounced effects on DNA methylation. Given the small number of samples in each group, the nature of the Illumina EPIC array data and considerations reflected in [19], these relaxed pairwise results must be interpreted with caution [19].

Preoperative & postoperative samples

Principal components analysis

Samples were processed and loaded similarly (Supplementary Figures 1 & 2). PCA indicated that postoperative samples were grouped separately from preoperative samples, which suggests a strong effect of the surgery. Figure 3 shows that both Illumina normalization (Figure 3A) and functional normalization with background adjustment and dye correction (as described in the Methods; Figure 3B) produced similar PCA results. The introduction of disease as a factor (Figure 3C) revealed that the disease-induced effects seen in preoperative samples are overpowered by the effects of CPB.

Analysis of variance

The preoperative findings on the weak effects of HTN and strong effects of DM on DNA methylation were no longer distinct in the postoperative sample set; therefore we compared these groups across all samples in the study (both preoperative and postoperative). We found that HTN alone affected 160 loci at a high stringency ($p < 0.05$ FDR, Benjamini adjusted), whereas controlled DM affected 5701 loci and uncontrolled/poorly controlled DM affected 6775 loci; there were no changes (six loci) between controlled and uncontrolled DM. The strong overall effect of DM and weak effect of HTN on DNA methylation is similar to our findings in the preoperative samples.

ANOVA interaction

ANOVA interaction analysis revealed 4537 loci significantly changed by both preoperative versus postoperative status and by disease (Figure 4A). The HCL dendrogram makes it evident that the differences by disease are masked in postoperative samples as compared with preoperative samples.

ANOVA interaction analysis was also used to determine the differential responses to CPB in each group by disease status. At the stringency of FDR-adjusted $p < 0.05$ and with a difference cutoff of ±2, we found that 1849 loci changed in control, 809 in HTN only, 181 in HTN+cDM and 181 in HTN+uncDM groups; notably, the latter two were the same genes. At a more relaxed unadjusted $p < 0.05$ with the same difference cutoff, we found that 2132 loci changed in control, 1034 in HTN only, 301 in HTN+cDM and 274 in HTN+uncDM groups. Therefore the 'control' SKM responded to CPB with changes in more loci than the HTN and HTN+DM SKM samples.

Paired t-test

The paired t-test comparing preoperative and postoperative methylation independent of disease status revealed 282 highly significant loci (Figure 4B) with M-value difference over ±2 and Benjamini FDR-adjusted $p < 0.05$. Of these loci, 45 were mapped to 5′ UTR or the first exon, four to the 3′ UTR, 105 to gene body, 62 to <1500 bp

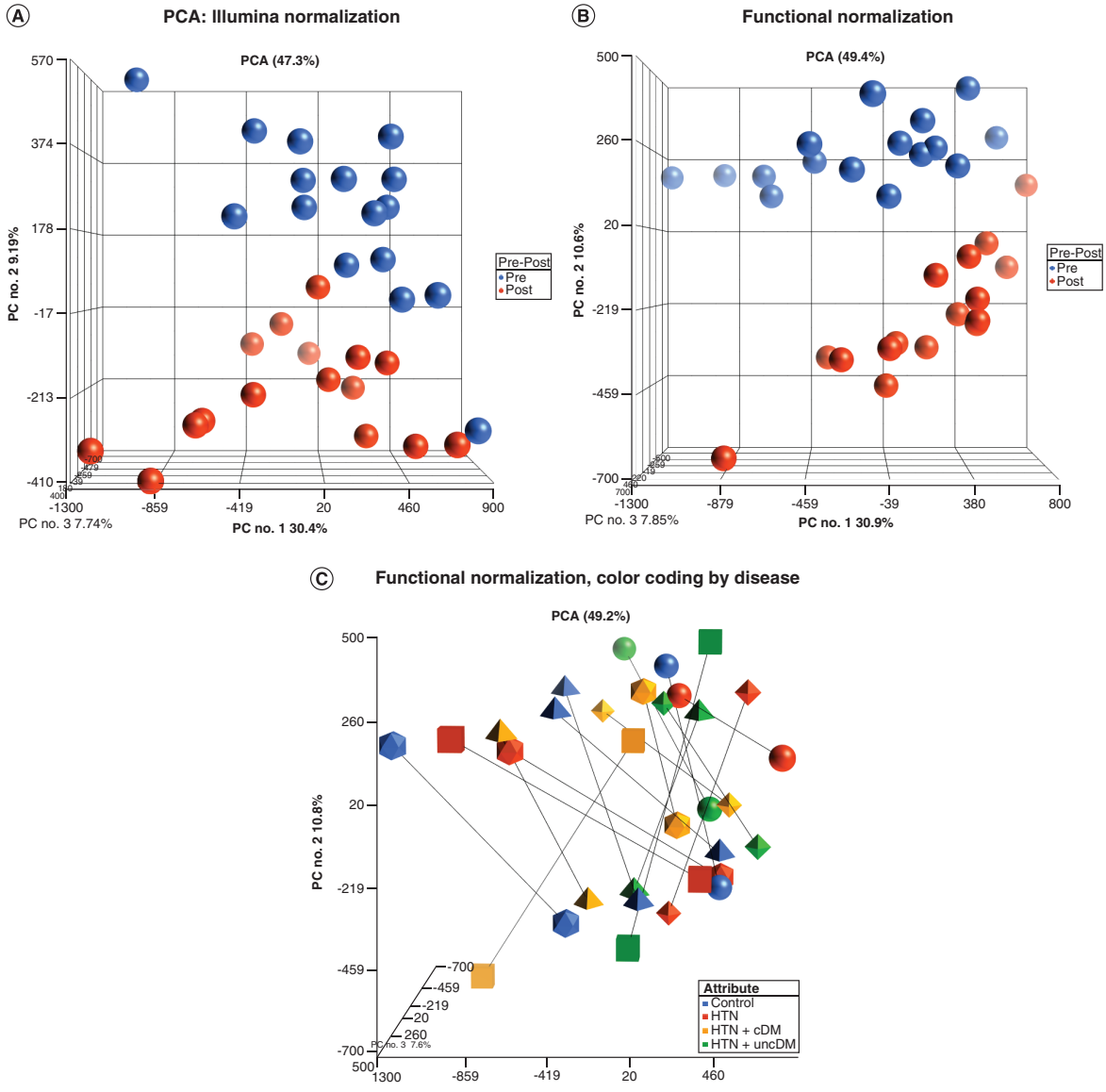

Figure 3. Principal components analysis of preoperative and postoperative samples. PCA showing methylation profiles of the study samples. Each sample is represented by a dot, the axes are the first three PCs, and the number in parentheses indicates the fraction of variance explained by each PC. The number at the top is the variance explained by the first three PCs. The samples are colored by study group: in **(A & B)** the comparison aims to highlight the effects of the surgery, while in **(C)** the colors highlight the effects of the disease. **(C)** Samples from each individual patient before and after surgery are connected by a line.
cDM: Controlled diabetes mellitus; HTN: Hypertension; PC: Principal component; PCA: Principal component analysis; uncDM: Uncontrolled diabetes mellitus.

from the transcription start site and the others to nearby intergenic locations. The analysis also revealed 193,567 loci with unadjusted $p < 0.01$; however, the magnitude of the effect did not exceed 10–20%, which suggests that CPB induced a few strong changes and a plethora of minimal effects on DNA methylation.

HCL analysis of this list supervised by preoperative versus postoperative status but unsupervised by disease further supports this study's prior finding that DM samples (HTN+cDM or +uncDM) are more dissimilar to non-DM samples. The HCL analysis also visualized an outlier in the postoperative set previously seen on PCA: as seen in Figure 4B, the top sample belongs to the HTN+uncDM group but does not cluster with it.

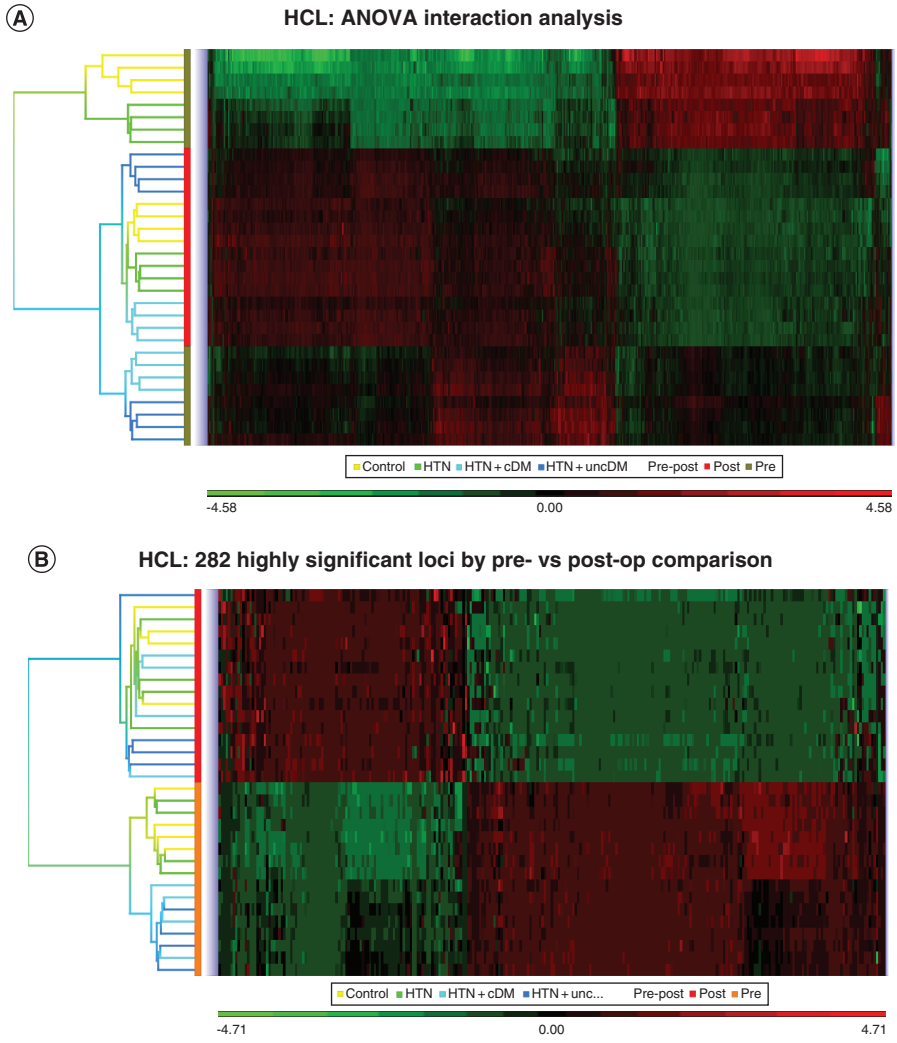

Figure 4. Analysis of preoperative and postoperative samples. (A) Hierarchical clustering of 4537 loci significantly changed by both preoperative versus postoperative status and by disease, as determined by analysis of variance interaction analysis. **(B)** Paired t-test comparing preoperative and postoperative methylation independent of the disease status revealed 282 highly significant loci with M-value difference over ±2 and Benjamini false discovery rate-adjusted $p < 0.05$. Hierarchical clustering of this list supervised by preoperative versus postoperative status but unsupervised by disease confirms the prior finding that diabetes samples (HTN+cDM or +uncDM) are more dissimilar to non-diabetes (HTN or control) samples.
cDM: Controlled diabetes mellitus; HCL: Hierarchical clustering; HTN: Hypertension; uncDM: Uncontrolled diabetes mellitus.

A list of 282 significant loci for postoperative versus preoperative comparison were used for network and pathway enrichment. A 'direct interactions' network analysis indicated a small network of inter-related genes focused mainly on the transcription factors *RUNX* and *E2F1*.

The pathway mapping feature did not suggest a substantial link to any pathway; those involved included three or four genes out of 50–70 in a typical pathway, suggesting that the 'hits' identified did not assemble within one or a few known pathways.

Broadening the criteria to include loci altered with at least 1.5 difference instead of 2.0 identified 941 loci. Network analysis of this list indicated a larger interconnected cluster, focusing again on *RUNX* and *E2F1* but also including *GABP*-α, PKA, *NF-AT*, importin and activin A receptor II as major nodes. Pathway mapping also became more enriched, showing the STK3/4 (Hippo) pathway and YAP/TAZ pathway, adenosine receptor signaling and a few other signaling pathways in addition to apoptosis and autophagy mechanisms (Supplementary Figure 6). The involvement of the following was also noted: protocadherin, tissue kallikrein, troponin C and

tropomyosin, fibronectin and MHC class II β-chain as well as HLA *DPB1, DPB2* and *DOB* genes (the latter might suggest an influx of immune cells into remote tissues). From the point of view of gene ontology, several hundred of these factors were associated with the term 'binding' or the binding of proteins, ions or metals. Analysis by chromosome suggested these effects occurred randomly throughout the karyotype.

Hippo–YAP/TAZ pathway

The top pathway highlighted by Metacore GeneGo in our high-stringency comparison of postoperative versus preoperative samples ($p < 0.05$ FDR-adjusted, difference $> \pm 1.5$) was the Hippo–YAP/TAZ pathway [20]. While these genes are involved in Hippo pathway signaling, they are not specific to this pathway, which led us to check the effects of the more specific genes traditionally comprising the Hippo–YAP/TAZ signaling system [20].

We then looked into a more relaxed list of postoperative versus preoperative effects based on unadjusted p-values and specifically at the factors of the Hippo pathway (see Supplementary data). We found altered methylation in a subset of loci associated with *YAP1, TAZ, Hippo, Warts, Salvador, Merlin* and *Scalloped* genes. While these changes were not pronounced, a few loci were highly significant and more loci were reasonably significant (see Supplementary data). Thus virtually every gene in the Hippo–YAP/TAZ pathway was affected, albeit to a variable and often small degree.

Discussion

Epigenomic studies of the SKM, particularly in humans, are rare, albeit those available suggest SKM responds to a variety of physiological and pathological conditions with epigenetic changes linked to altered gene expression [21]. The muscle adapts to physiological challenges including aging [22] and exercise [23], and some of this adaptation involves epigenetic mechanisms. The SKM epigenome has been shown to be affected in a few diseases, including diabetes, and epigenetic alterations may be linked to impaired rehabilitative plasticity [24,25]. Less is known about epigenomic changes in SKM occurring in patients with HTN, although other tissues and cells have been investigated [26,27]. In general, few studies have looked into tissue-specific epigenetic patterns in SKM [28]. Investigating alterations in the SKM epigenome is important, however, as reports suggest that it may be sensitive to even short-term challenges [29].

Our PCA, ANOVA and LIMMA analyses of preoperative samples suggest that DM with HTN is a more powerful trigger of epigenomic changes than HTN alone. Even though we did not have a 'DM alone' group, the lack of changes in HTN alone versus control suggests that the 'metabolic' condition (DM) may affect the SKM epigenome to a greater extent than the 'hemodynamic' condition (HTN). If true, this is consistent with the lack of published data on the effects of HTN on the muscle epigenome in contrast to available epigenomic effects of DM [11,12,30–32]; however, a direct comparison of DM alone versus HTN alone in a larger cohort would be needed to directly address this question. Our network and pathway analysis does not provide a ready answer to the biological processes likely affected, but our results suggest that the altered loci are associated with genes that mediate signaling in immune and inflammatory pathways, apoptosis, DNA damage response and metabolic processes including protein synthesis and cell proliferation.

We hoped in part that our analysis of postoperative versus preoperative samples could identify a simple metric as an outcome of surgery, such as a change in global DNA methylation. However, as often happens in epigenomic studies of responses to environmental/injurious triggers, the epigenetic changes occurred in both directions. Our analysis suggests that, first, the overall methylome (without looking into significant DMLs) does not change in either direction (Supplementary Figure 1) because the majority of CpG loci are not altered; and second, that among the significant DMLs, changes occur in both directions in approximately equal numbers, rather than producing substantially more demethylated or more hypermethylated loci overall. This may be in contrast to global uni-directional epigenome-wide effects like those of DNMT inhibitors. We argue that intricate details of changes in the epigenetic landscape that occur in response to milder insults, like the one here, provide justification for 'detailed' epigenomic profiling rather than 'global' DNA methylation analysis methods.

SKM appears to have 'epigenetic memory', previously reported as a positive result of exercise [33,34]. One can speculate that a similar negative effect may occur as a result of stress, insult or injury, such as the effects induced by CPB during cardiac surgery; although we did not study it here, such epigenetic 'scarring' may be linked to postoperative recovery and may help explain some of the difficulty associated with regaining muscle function [35,36]. Interestingly, DNA methylation changes in SKM linked to metabolic syndrome have been reported to transmit maternally to progeny [37].

Seeing epigenetic effects from short-term stressors like a coronary artery grafting surgery lasting only 3–6 h is not unique [38,39]. While some studies reported that a matter of hours may not be sufficient to induce epigenetic change [40], some loci in SKM can undergo remarkably rapid adaptive methylation changes [41]. Similarly quick DNA methylation changes were found in neurons of rats [42] and humans [43] and in peripheral blood monocytes after surgery [7]. Further studies are needed to investigate whether these effects would persist into the long term. Furthermore, studies comparing SKM DNA methylation changes of a surgery involving CPB versus those of a similar operation without it are needed to better understand the mechanism of DNA methylation changes. CPB has multiple potential mechanisms by which it can trigger a systemic inflammatory response, including activation of immune and endothelial cells [44]; a prolonged surgery with anesthesia and infusions could have similar effects. Others have reported that surgery with CPB can have epigenetic implications [45–47]; however, whether these effects are CPB-specific remains unknown. To provide insight into the matter, we checked whether there was correlation between β-values and CPB time for the 941 significantly altered DMLs. The CPB times ranged from 55 to 349 min, with an average per group of 76.5–87.5 min (Supplementary Tables 2 & 3). Correlation analysis revealed that only 10 out of 941 DMLs correlated with CPB time (∼1% of significantly altered loci). We interpret this to mean that while methylation changes in some loci may be linked to CPB time, the majority of loci were not correlated. This does not mean that CPB did not contribute to the effects; it is possible that after some duration sufficient to launch the response processes leading to the methylome effects we observed, a longer time merely does not further increase the magnitude of methylation changes.

Hippo–YAP/TAZ is an evolutionarily conserved pathway involved in organ growth and development, amplification of tissue-specific progenitor cells during tissue renewal and regeneration, and angiogenesis [20,48]. This pathway responds to changes of cell shape and tension and other mechanical challenges [20]. Therefore, while further targeted studies are needed to determine the true extent of methylation changes in Hippo–YAP/TAZ pathway genes, our data could support the idea that epigenetic changes in the genes of this pathway may represent a rapid response to injury that occurs during CPB.

In general, the epigenetic effects of surgery we identified could occur both as a consequence of damage/injury and as part of an adaptation process linked to modified gene transcriptional activity. A surgery under general anesthesia and involving CPB has a number of chemical, mechanical, metabolic, hemodynamic and other effects with inflammatory/cytotoxic action that entail changes in gene regulation. We will continue to investigate the causes for the methylation changes in ongoing research.

We note another possible contributing factor stemming from the fact that we had no control of the cellular content in our muscle specimens. For example, our finding of HLA molecules among those with altered methylation may be explained by monocyte/macrophage or other immune cell influx into the muscle during CPB and not by epigenetic changes in the myocytes themselves [49]. Another aspect to consider is that different muscle fibers have differences in epigenomic profiles [50,51]. It is important to note, however, that this is unlikely to be a factor over such a short period of time as in our study.

Interpreting tissue-level epigenomic data poses its own challenges [52]. Nevertheless, SKM tissue is substantially more homogenous than, for example, lung or brain tissue. Our bioinformatic strategy was aimed at identifying loci with significant and substantial change that was unlikely to be driven by a small variation of cellular content. We point out that tissue-level epigenomics can indeed be a source of valuable mechanistic insights [41,53].

Various potential confounders could not be accounted for in our design. The severity of HTN, glucose levels and other factors like medication or exercise vary across patients over time. There was individual variability in CPB time and the amount of surgical work performed on each patient. We also did not stratify the patients by age, sex, body mass index or other similar parameters. We disregarded these details in favor of a 'proof-of-concept' study aimed at determining whether detectable methylation changes do occur after a relatively short operative procedure. However, even with this potential heterogeneity, we were able to identify the significant DMLs that were present in all patients of some, but not other groups. Hence, while further studies are needed to determine the individual inputs of blood pressure, glucose and surgical parameters, we believe our work is useful to characterize rarely reported rapid changes in the SKM methylome.

We did not use a stratified randomization design with regard to sample position on a chip, partly because this exploratory study was conceived to identify disease (HTN and DM) effects in preoperative samples first, followed by postsurgical testing later. In order to address potential chip-related and positional bias, Illumina EPIC chips employ a set of internal controls that allow normalization with background adjustment that minimizes such unwanted effects. To avoid batch effects we performed the analysis of disease effects in preoperative samples

separately, so that the first set of results (those related to the effects of HTN and DM) is based on preoperative samples only. Only then did we combine all chips (preoperative and postoperative samples) into one dataset and performed an all-through normalization with background adjustment across all chips to minimize the batch effect. We relied on either Illumina 'built-in' background adjustment and normalization methods or on 'functional normalization' [14], which is a Bioconductor minfi package normalization, with NOOB background correction (out-of-band normalization) and dye correction [15], and found minor but not substantial differences between the two normalization techniques. We therefore opted for the functional normalization method with NOOB. We feel obligated to mention that array studies always entail a potential for positional effects (e.g., chip-, and even, row-effects); hence the interpretation of small exploratory studies like ours should be done in the context of larger studies with more samples that might hopefully follow.

Finally, due to the small number of patients in the preliminary study, we could not correlate epigenomic changes with clinical outcomes, which will be a future direction in understanding the importance of the gene-level effects of cardiac operations. We have made the epigenomic signatures of HTN and DM in human SKM publicly available (see NCBI GEO GSE173613), hoping these data will help inform future studies.

A benefit of an 'omics' approach is often that it helps generate new hypotheses. Based on our findings, we can speculate that further studies may be able to establish and better define the 'epigenomic scar' caused by surgery. Our data may lead to a hypothesis that activation of tissue repair mechanisms occurs rather quickly after injury and involves epigenetic regulation. Our data may also prompt investigations into potential damaging factors (e.g., chemical, immunological, mechanical, hemodynamic) that act via yet unknown epigenetic mechanisms during surgical procedures.

Conclusion

Hypertensive patients with DM, whether well controlled or poorly controlled, show substantial changes in SKM DNA methylation across the epigenome. HTN alone did not influence the methylome as significantly as DM in the muscle. During CPB, multiple low-grade methylation changes occur that mask the effects of the disease. Moreover, more prominent changes occur in a few loci that may be part of a compensatory/regulatory response to surgery.

Summary points

- A combination of diabetes and hypertension is associated with epigenomic changes in the skeletal muscle which are more pronounced than those associated with hypertension alone.
- A heart surgery with cardiopulmonary bypass induces epigenomic change in skeletal muscle. Here we show that even in the short time taken for a cardiopulmonary bypass operation, multiple epigenome-wide DNA methylation changes occur in skeletal muscle.
- Most of these changes appear mild but broad; however, a few are pronounced.
- The effects of surgery on epigenetic regulation have been largely neglected. This may be a target for postoperative evaluation and therapeutic correction.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2021-0388

Author contributions

G Aghagoli, A Del Re, Z Zhang, AA Gheit, R Phillips and F Sellke participated in surgery, recruited patients and collected patient samples. G Aghagoli and A Del Re identified patient samples and analyzed patient information. G Aghagoli co-ordinated the study and was in charge of sample storage and transportation. N Yano processed samples and isolated DNA, performed quality control and validation. F Sellke and A Fedulov conceived and supervised the study. F Sellke led the surgical team and performed surgeries and postoperative care. A Fedulov processed DNA samples and analyzed data. G Aghagoli, A Del Re, N Yano, F Sellke and A Fedulov coauthored the manuscript. All authors participated in drafting and/or revising the paper, provided important intellectual contributions and approved the manuscript.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval form Rhode Island Hospital Institutional Review Board (IRB no. 225612-53) for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

The data informing this manuscript are publicly available through NCBI GEO database (GSE173613).

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